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A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates

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1. A new method has been developed for measuring the total antioxidant capacity of body fluids and drug solutions, based on the absorbance of the $ABTS^{\cdot+}$ radical cation.
2. An automated method for use on a centrifugal analyser, as well as a manual method, is described.
3. The procedure has been applied to physiological antioxidant compounds and radical-scavenging drugs, and an antioxidant ranking was established based on their reactivity relative to a 1.0 mmol/l Trolox standard.
4. The Trolox equivalent antioxidant capacity of plasma from an adult reference population has been measured, and the method optimized and validated.
5. The method has been applied to investigate the total plasma antioxidant capacity of neonates and how this may be compromised in prematurity.

INTRODUCTION

On exposure to hydrogen peroxide, metmyoglobin and methaemoglobin are activated to ferryl states [1, 2] in which the iron is one oxidizing equivalent above the original level and one oxidizing equivalent is on the surface of the protein [1]. With reducing agents (electron or hydrogen donors) these species are reduced back to metmyoglobin or methaemoglobin [2–4].

In this study we have exploited the peroxidase activity of metmyoglobin combined with its interaction with a phenothiazine compound to form a radical cation intermediate [5], to establish a method for measuring antioxidant status [6]. The method derives from the observation that when 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) is incubated with a peroxidase (such as metmyoglobin) and hydrogen peroxide, the relatively long-lived radical cation, $ABTS^{\cdot+}$ [7], is formed. A large number of free radicals, such as hydroxyl, peroxyl, alkoxyl and inorganic radicals, also react rapidly with ABTS to form this species [8]. When the peroxidase is metmyoglobin, the formation of the $ABTS^{\cdot+}$ radical cation on interaction with ferryl myoglobin has absorption maxima at 650, 734 and 820 nm, beyond the region of absorption of the haem proteins. In the presence of antioxidant reductants and hydrogen donors, in pure solution or in plasma, the absorbance of this radical cation is quenched to an extent related to the antioxidant capacity of the added fluid (in the absence of interfering compounds).

The major antioxidant defences in plasma include ascorbate, protein thiols, bilirubin, urate and α-tocopherol. The 'chain-breaking' or 'radical-scavenging' agents against oxidative stress are considered to act in the above sequence of increasing to decreasing effectiveness against free radicals generated in the plasma aqueous phase [9]. Plasma also contains the 'preventive' antioxidants, caeruloplasmin and transferrin, the iron-scavenging proteins whose contribution to the total antioxidant capacity is to prevent iron availability [10].

The limited ability of pre-term infants to cope with oxidative stress may contribute to the development of broncho-pulmonary dysplasia, necrotizing enterocolitis and retrolental fibroplasia. The possible role of antioxidants in protecting against oxygen toxicity has been inconclusively investigated for a number of years. Pre-term infants have incompletely developed antioxidant defences and suffer from a deficiency of vitamin E, which is normally derived at the end of the third trimester from the maternal circulation.

Applying this method, the total plasma antioxidant status of pre-term babies has been monitored and compared with that of term babies and maternal levels. The results show that infants born at 27 ± 2 weeks have a significantly depressed total

Key words: antioxidant, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid), ferryl myoglobin, free radicals, premature neonates, Trolox.

Abbreviations: ABTS, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid); TEAC, Trolox equivalent antioxidant capacity; TRAP, total peroxyl radical-trapping antioxidant parameter.

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plasma antioxidant activity at birth. The differential values between pre-term and term babies are reflected in the maternal levels, supporting not only the need for antioxidant supplementation of preterm babies from birth, but also the monitoring of maternal blood levels during pregnancy.

MATERIALS AND METHODS

Materials

Myoglobin type III, iron in ferric state, L-ascorbic acid, uric acid, albumin (human fraction V), α-tocopherol, glutathione, bilirubin, EDTA (disodium salt), heparin (sodium salt) and Sephadex G-15-120 (40–120 μm), were purchased from Sigma; ABTS (diammonium salt) and Trolox (registered trademark of Hoffman–LaRoche) were purchased from Aldrich; hydrogen peroxide, disodium hydrogen orthophosphate 12-hydrate, sodium dihydrogen orthophosphate dihydrate, sodium chloride, urea, creatinine, glucose, mannitol and potassium ferricyanide were obtained from BDH. Desferrioxamine (Desferal) was obtained from Ciba–Geigy. Freeze-dried serum quality control pools were obtained from Biostat.

Metmyoglobin was purified after adding the stock myoglobin solution (400 μmol/l) in 5 mmol/l isotonic PBS (pH 7.4) to an equal volume of freshly prepared 740 μmol/l potassium ferricyanide; after mixing, the solution was passed through a G15-120 Sephadex column equilibrated in the buffer, and the metmyoglobin fraction was collected. The final concentration of purified metmyoglobin was estimated by applying the Whitburn equations:

$$[\text{Met Mb}] = 146A_{490} - 108A_{560} + 2.1A_{580}$$

$$[\text{Ferryl Mb}] = -62A_{490} + 242A_{560} - 123A_{580}$$

$$[\text{MbO}_2] = 2.8A_{490} - 127A_{560} + 153A_{580}$$

where Mb is myoglobin. These equations [11] are derived by solving simultaneous equations based on Beer's law, measuring the absorbance at 490, 560 and 580 nm, and subtracting the reading at 700 nm to correct for background absorbance. The purity of the metmyoglobin prepared was estimated by applying all three equations. Normally the metmyoglobin fraction is >95% of the total haem protein.

A 2.5 mmol/l Trolox solution was prepared by dissolving 0.15643 g of Trolox in 250 ml of buffer. Dissolution was facilitated by gentle sonication. α-Tocopherol was solubilized for introduction into the assay system by preparing an emulsion in 60% ethanol/40% water containing 2% Nonidet P-40.

ABTS was prepared as a 5 mmol/l solution by dissolving 0.02743 g in 10.0 ml of buffer; 500 μmol/l ABTS was prepared from this stock solution for use as a working reagent. Hydrogen peroxide was diluted to a 0.45 mmol/l working solution.

Spectroscopic assay

ABTS (300 μl, 500 μmol/l), 36 μl of metmyoglobin (70 μmol/l) and 497 μl of buffer (of which 8.4 μl was replaced when a sample was being investigated) were mixed, and the reaction was initiated by the addition of 167 μl of hydrogen peroxide (450 μmol/l), giving final concentrations of 2.5 μmol/l metmyoglobin, 150 μmol/l ABTS amd 75 μmol/l hydrogen peroxide. Spectra were recorded (see Fig. 1) at 90 s intervals for 12 min (450–900 nm), and the absorbance at 734 nm was measured as a function of time.

Centrifugal analyser assay

For the assay to be carried out on the Roche Cobas Bio centrifugal analyser, the final incubation volume in the cuvette and the method of making additions required that reagents be made up in concentrations that suited the configuration of this instrument. In the protocol devised, 300 μl of ABTS/myoglobin reagent was mixed with 3 μl of sample and a further 30 μl of diluent that flushed the sample probe. The initiator of the reaction, hydrogen peroxide (1.078 mmol/l), was added last (25 μl) to give a final concentration in the cuvette of 75 μmol/l and a final incubation volume of 358 μl.

A quantitative relationship exists between the absorbance at 734 nm at 6 min and the antioxidant concentrations of the added sample or standard. After 6 min the absorbance of the solution was read at 734 nm, along with a buffer blank that did not contain the antioxidant solution or fluid to be tested. The absorbance value of the blank at 734 nm at 6 min minus that for the test solution, divided by the blank value, was the fractional inhibition of the reaction.

Patients

Babies were recruited at the time of delivery when cord venous blood samples were collected. Further venous blood samples (0.5 ml) were obtained on day 5 in term babies at the time of the Guthrie test and in pre-term babies at the time of routine blood sampling.

Term babies (39–41 weeks gestation) weighed 2.20–3.92 kg; pre-term babies (25–29 weeks gestation) weighed 0.73–1.46 kg. Three of the premature babies subsequently died of their complications (intraventicular haemorrhage, group B streptococcal sepsis, necrotizing enterocolitis) and three developed bronchopulmonary dysplasia. The remaining ten premature infants did well after their initial period of ventilation. Each premature infant was treated with surfactant (Exosurf), which showed no activity when introduced into the antioxidant assay as a sample, and penicillin and gentamicin, which both showed moderate antioxidant activity. None of

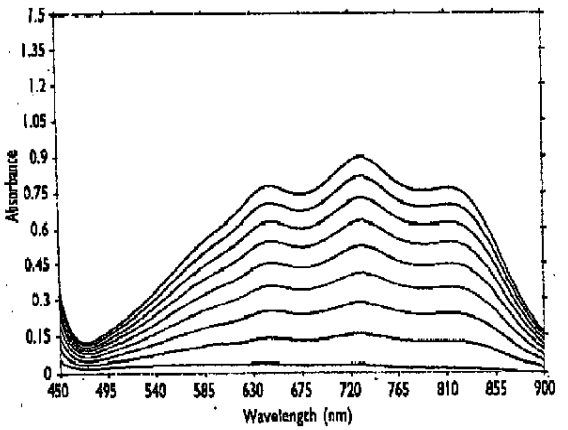

Fig. 1. Visible spectra recording the appearance of the $ABTS^{\bullet+}$ radical cation with time. Scans were run at 90s intervals commencing at 15s. Conditions: 2.5 μmol/l metmyoglobin, 150 μmol/l ABTS and 75 μmol/l H_2O_2.

these substances could therefore act as negative interferents in the antioxidant assay.

RESULTS

Incorporation of a range of individual antioxidants (initial concentration from 0.25 to 10.0 mmol/l) before the addition of hydrogen peroxide induced a delay in the appearance of the $ABTS^{\bullet+}$ radical cation and hence increased the percentage inhibition of the absorbance at 734 nm (Fig. 1). All the following results described were derived from analyses carried out on the Cobas Bio centrifugal analyser.

Standardization of the assay

The system was standardized using Trolox, an α-tocopherol analogue with enhanced water solubility. A 2.5 mmol/l solution of Trolox was prepared in buffer. This solution is stable for 1 week when stored at 4°C or for 6 months when stored at −20°C. Standards of lower concentration were prepared fresh daily.

The results for dose–response curves derived from 16 sequential and separately prepared stock standards are summarized in Fig. 2. The SD of the 0.5 and 2.5 mmol/l points is less than 2.0, and so falls within the area of the plotted point. The dose–response curve is thus highly reproducible.

Units of measurement: Trolox equivalent antioxidant capacity (TEAC)

The unit of activity is the TEAC, which is defined as the concentration (mmol/l) of Trolox having the equivalent antioxidant capacity to a 1.0 mmol/l solution of the substance under investigation. The deri-

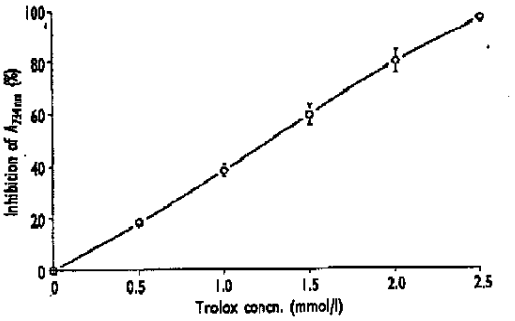

Fig. 2. Degree of inhibition of the absorbance at 734 nm as a function of the concentration of Trolox. Values are means ± SD ($n = 16$).

Table 1. TEAC values

	TEAC	n	SD
Desferrioxamine	2.96	4	0.09
Bilirubin	1.50	3	0.12
Urate	1.02	5	0.06
Ascorbate	0.99	5	0.04
α-Tocopherol	0.97	3	0.01
Glutathione	0.90	3	0.03
Albumin	0.63	3	0.02
EDTA	0.05	3	0.01
Mannitol	0.00	3	
Glucose	0.00	2	
Ethanol	0.00	2	
Heparin	0.00	5	
Urea	0.00	3	
Creatinine	0.00	3	

vation of a TEAC value provides a method for comparison of antioxidant activity among groups of drugs and chemicals, provided that they are water-soluble or can be solubilized. The results are shown in Table 1. Whereas urate, α-tocopherol and ascorbate are as effective as Trolox in their antioxidant activity, desferrioxamine is three times more effective as a scavenger. Bilirubin is more efficacious than these compounds, whereas albumin is considerably less active.

Total antioxidant activity of normal human plasma

Incorporation of plasma from an adult reference population as samples into the assay gave a 0.95 interfractile interval of 1.46 ± 0.14 mmol/l ($n = 312$).

Precision

Two control pools were prepared from commercial freeze-dried quality control sera. The results for intra-assay precision when analysed repeatedly in a single assay, or inter-assay precision, obtained by re-analysing the pools on 20 sequential days, are shown in Table 2. The data indicate the ability to

Table 2. Estimates of analytical performance

	High pool	Low pool
Intra-assay precision		
No.	25	25
Mean	1.52	0.83
SD	0.008	0.013
Coefficient of variation (%)	0.54	1.59
Inter-assay precision		
No.	20	20
Mean	1.52	0.84
SD	0.055	0.050
Coefficient of variation (%)	3.6	6.1

carry out the assay on a day-to-day basis with an acceptable degree of precision.

Interferences

The only true interfering substances in this assay are peroxidases, such as haem proteins, which, in the presence of hydrogen peroxide, promote the formation of the $ABTS^{\bullet+}$ radical cation. Visible haemolysis thus makes plasma samples unsuitable, but a study of delay in centrifugation and of *g* force applied during centrifugation showed that more minor levels of haemolysis do not affect the results.

Drugs and other exogenous materials with significant absorption at 734 nm might also be potential sources of positive interference in the assay.

Specimens from neonates and their mothers

Applying the method to monitoring the total plasma antioxidant activity of neonates, the findings reveal that the total plasma antioxidant status of pre-term infants at birth is significantly depressed (1.21 ± 0.08 mmol/l, $n=16$) compared with term babies (1.46 ± 0.07 mmol/l, $n=18$). After 5 days the antioxidant activity in the plasma of the premature infants had risen (1.41 ± 0.09 mmol/l, $n=14$), whereas that of the term infants showed no significant change. The differential values between pre-term and term babies were reflected in the related maternal blood levels of the small number of pair-matched samples studied so far [pre-term 1.17 ± 0.06 mmol/l ($n=6$) to maternal 1.25 ± 0.08 mmol/l ($n=6$); and term 1.46 ± 0.07 mmol/l ($n=18$) to maternal 1.41 ± 0.07 mmol/l ($n=17$)], demonstrating the influence of the maternal total antioxidant levels.

DISCUSSION

The derivation of a TEAC value, as shown above, provides a method for the comparison of antioxidant activity among groups of drugs, provided they are water-soluble (or can be solubilized), or body fluids, such as plasma. The method thus has important pharmacological and nutritional applications.

Table 3. Antioxidant hierarchy in the plasma water. A ranking of endogenous antioxidants, based on TEAC × mid-point of the plasma reference interval.

	TEAC	Concn. (μmol/l)			Antioxidant activity (% of total plasma activity)
		Range	Mid-point	TEAC × mid-point concn.	
Albumin	0.63	535–760	640	403	43
Urate	1.02	180–420	300	306	33
Ascorbate	0.99	34–111	73	73	9
α-Tocopherol	0.97	14–44	29	28	3
Bilirubin	1.50	<20	10	15	2
Unmeasured antioxidants					10

Applying this method, urate, α-tocopherol, ascorbate and glutathione have the same molar equivalent antioxidant capacity as Trolox. Of the other antioxidants found in human plasma, bilirubin is a more efficient antioxidant and albumin is less active, on a mol for mol basis. Desferrioxamine, the therapeutic iron chelator, has been identified as a hydrogen-donating antioxidant to the superoxide radical [12], activated peroxidases [13] and ferryl myoglobin radical species [3, 4]. This compound has a three-fold greater activity in the assay as compared with urate, ascorbate, etc. which is as would be predicted on the basis of its trihydroxamate structure. The value derived for bilirubin was of the order of 1.5 Trolox equivalents. EDTA (10 mmol/l), heparin (10 000 i.u.), urea (10 mmol/l), creatinine (10 mmol/l), glucose (10 mmol/l) and ethanol (60%) were all unreactive in the assay at the concentrations applied. (The results for EDTA and heparin indicate their lack of interference as anticoagulants for plasma studies.) Cysteine has a low TEAC value and, under most circumstances, a very low concentration in the plasma. Glutathione is an active antioxidant with a TEAC value equivalent to that of urate and ascorbate. With one exchangeable hydrogen per mol of glutathione, this result is as predicted. Glutathione is unlikely to play a significant role in the extracellular fluid. The extremely hydrophobic nature of β-carotene, dihydrolipoate and ubiquinone makes it difficult to evaluate them in an aqueous system such as the one we have described.

Although the plasma is not a simple chemical system as regards antioxidant activity (the different antioxidants may be active at specific sites and hence perform special functions) one can extrapolate from the above to a ranking of antioxidants in the plasma water. This is shown in Table 3. The relative efficacy of the contribution of each antioxidant does not define the actual importance of the antioxidant because of variations in the plasma concentrations. Albumin is first in this ranking, due to its relatively

high concentration in the plasma water, even though it is a comparatively less efficient antioxidant. For purposes of comparison, albumin concentrations in Table 3 are expressed in molar units (applying an M_r of 65000 [14]), although this is not the common practice. Uric acid ranks second to albumin, again due to its high concentration. More than 70% of the antioxidant activity of the plasma water is accounted for by these two substances.

The previously published [15] total peroxyl radical-trapping antioxidant parameter (TRAP) is based on the application of the thermal decomposition of the water-soluble azocompound 2,2'-azo-bis-(2-amidinopropane hydrochloride) to peroxyl radicals. The improved method from these workers [16] applied to plasma samples shows contributions from urate ($58 \pm 18\%$), plasma proteins ($21 \pm 10\%$), ascorbate ($14 \pm 8\%$) and vitamin E ($7 \pm 2\%$) to TRAP. The residual contribution after accounting for urate and 'plasma proteins', in these authors' terminology, is equivalent to the residual antioxidant activity presented here after accounting for albumin and urate.

Lindeman et. [17], applying the TRAP assay of Wayner et al. [16], have determined the percentage contributions to the measured TRAP as: uric acid, 47.4%; thiol (plasma proteins), 12.2%; vitamin C, 13.4%; vitamin E, 6.4%; unidentified substances, 21.5%. Whereas uric acid, vitamin C and vitamin E are reasonably close to our estimate, some differences are apparent. We deduce that the thiol measure used by these workers is that in the plasma proteins, i.e. mainly albumin, for which they quote a plasma concentration of 496 μmol/l. We have calculated from a reference interval for albumin of 35–50 g/l [18] (which converts to 535–760 μmol/l). The resulting underestimate could account for the higher value quoted for unidentified compounds, as compared with our value of 10%.

Albumin is well recognized as a plasma expander, through its maintenance of the plasma oncotic pressure. It is also regarded as an important non-specific transporter of endogenous substances, drugs and toxins. The importance of albumin as a free-radical scavenger apart from these two other functions has been reported [19]. There is also some epidemiological evidence for its importance as an antioxidant: a consistent negative relationship between albumin and coronary heart disease is known [20]. The importance of uric acid is also well recognized [21]. These two substances may act as a general pool for the removal of radicals passed in the plasma water. Ascorbate itself accounts for just under 10% of the total plasma antioxidant activity, and its high renal and hepatic clearance makes it unlikely that this value could be greatly raised. Bilirubin, like uric acid, may prove to be an important antioxidant in certain pathological situations where levels in the plasma rise well above the reference interval. The remaining 10% of the total plasma antioxidant activity is accounted for by substances such as cysteine and glutathione, which are water-soluble, and β-carotene, dihydrolipoate and ubiquinone, which are non-polar hydrophobic substances.

Pre-term infants have incompletely developed antioxidant defences and suffer from a deficiency of vitamin E, which is normally derived from the maternal circulation at the end of the third trimester. Karmazsin et al. [22] have demonstrated a reduction in the plasma antioxidant activity of premature babies, using the bovine brain homogenate methods of Stocks et al. [10]. Oxygen therapy reduces the levels of free-radical scavengers in the extracellular fluid. It has been proposed that this state of affairs may predispose to free-radical damage. Vitamin E therapy has been shown to be beneficial in this event [23]. However, two double-blind controlled studies failed to show that vitamin E administration reduced the incidence of bronchopulmonary dysplasia in pre-term infants and may indeed increase the babies' susceptibility to infection by reducing the oxygen-dependent ability of neutrophils to kill pathogenic bacteria [24].

Other workers [17] have measured the TRAP of cord blood from babies at 32 weeks gestation compared with that of term babies and peripheral venous plasma from adults; their study revealed no significant differences, although this probably related to the degree of prematurity of the neonates.

Our results, using serial plasma samples from babies of less than 32 weeks gestation and their mothers, support the contention that such infants are profoundly antioxidant-deficient at birth. Monitoring maternal antioxidant levels during pregnancy where indicated may also be ultimately beneficial to the neonate.

Studies are in progress to identify patient groups among whom the measurement of total antioxidant activity in body fluids may prove to be an important prognostic or diagnostic guide. These include patients with atherosclerosis, reperfusion injury, septic shock and diabetes.

Thus we have presented an assay for measuring total plasma antioxidant status, which can be automated and which uses only 3 μl of plasma. The new method may be marginally less rigorous than the TRAP assay as it involves a number of reactive species rather than just the chain-carrying peroxyl species. However, the TRAP assay is manual and time-consuming. The new method presented here is extremely simple and rapid to perform, includes multi-point calibration of the dose–response curve, and is suitable for the analysis of large numbers of samples.

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